DermaVir: A Novel Topical Vaccine for HIV/AIDS

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Human immunodeficiency virus (HIV) vaccines have the potential to improve antiretroviral drug treatment by inducing cytotoxic killing of HIV-infected cells. Prophylactic vaccines utilize new antigens to initiate immunity; however, in HIV-infected individuals the load of viral antigen is not the limiting factor for the restoration of immune responses. Here we describe a novel immunization strategy with DermaVir that improves viral antigen presentation using dendritic cells (DC). DermaVir contains a distinctive plasmid DNA expressing all HIV proteins except integrase to induce immune responses with broad specificity. The DNA is formulated to a mannosilated particle to target antigen-presenting cells and to protect the DNA from intracellular degradation. After topical application, DermaVir-transduced cells migrate from the skin to the draining lymph node and interdigitate as DermaVir-expressing, antigen-presenting DC. We compared the immunogenicity of topical and *ex vivo* DC-based DermaVir vaccinations in naïve rhesus macaques. Both vaccinations induced simian immunodeficiency virus-specific CD4 helper and CD8 memory T cells detected by an *in vivo* skin test and an *in vitro* intracellular cytokine-based assay. Topical DermaVir vaccination represents an improvement upon existing *ex vivo* DC-based immunization technologies and may provide a new therapeutic option for HIV-infected patients.

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One strategy for a new immunotherapeutic intervention against human immunodeficiency virus (HIV) infection is to develop a vaccine that can reconstitute HIV-specific immunity, thereby improving the efficacy of the present antiretroviral regimens. The therapeutic efficacy of such a vaccine would be mediated by HIV-specific T cells, which play a central role in the control of virus replication (Amara and Robinson, 2002; Lisziewicz *et al*, 2003). Therapeutic vaccines are conceptually different from preventive vaccines. Preventive vaccines must prime the immune system with foreign viral antigens to induce immune responses that protect individuals from infection. In contrast, HIV-infected individuals have already developed immune responses to HIV, although insufficient to fully suppress virus replication in the majority of patients. In these subjects, HIV is expressed in most organs and viral load exceeds several thousands of viral RNA copies per milliliter in the peripheral blood. Consequently, the load of viral antigen is not the limiting factor in the induction of potent immune responses. Since it is unlikely that additional viral antigen would provide therapeutic benefit, we focused on the improvement of antigen presentation in order to induce functional HIV-specific T cells that could destroy HIV-infected cells.

We developed a new DNA-containing vaccine, called DermaVir, that presents viral antigens by dendritic cells (DC), the most potent type of antigen-presenting cell (APC) (Banchereau and Steinman, 1998), to improve antigen presentation during therapeutic immunization of chronically HIV-infected individuals. We have previously described an *ex vivo* vaccination strategy that uses DermaVir-transduced autologous monocyte-derived DC for the induction of HIV-specific T cells (Lisziewicz *et al*, 2001), and others have recently shown control of simian immunodeficiency virus (SIV) by *ex vivo* DC-based vaccination (Lu *et al*, 2003). DC-based vaccines using *ex vivo* cell manipulation are, however, inherently limited by their cumbersome nature. To improve upon this technology, we explored APC in the skin and their ability to deliver the vaccine *in vivo* to the secondary lymphoid organs.

The skin is a physical barrier between the internal milieu and the external world, the latter including its pathogens. The epidermis contains a large number of Langerhans cells (LC) (400–1,000 per mm^2), which take up and process epicutaneous antigens and migrate to the draining lymph nodes. Although in transit, they begin to differentiate into DC that present the processed antigens to naïve T cells (Steinman *et al*, 1995, 1997). The transfer of antigens to the lymphoid organs is critical for the induction of immune responses (Zinkernagel *et al*, 1997). Immune responses are

Abbreviations: APC, antigen-presenting cell; DC, dendritic cell; DTH, delayed-type hypersensitivity; HIV, human immunodeficiency virus; IFN, interferon; LC, Langerhans cells; PBS, phosphate-buffered saline; SHIV, simian human immunodeficiency virus; SIV, simian immunodeficiency virus

initiated in these lymphoid tissues, where antigen-loaded DC encounter and activate T cells (Steinman, 1991; Lanzavecchia and Sallusto, 2000). T cell activation results in the generation of both effector and memory cells that play distinctive roles against viral infection (von Andrian and Mackay, 2000; Masopust *et al*, 2001; Reinhardt *et al*, 2001). Therefore, LC in the epidermis might be used as a vehicle to transfer antigens from the skin to the lymphoid organs.

For the treatment of HIV/AIDS, we have improved the *ex vivo* DC vaccination technology with topical administration. DermaVir is administered to the surface of the skin with the aim of targeting epidermal LC and transferring DNA into these APC for presentation of viral antigens to T cells in order to induce new, functional HIV-specific T cells. The rationale of this design is based on the following observations: (i) early treatment of primary HIV/SIV infection, where DC are the major APC (Rowland-Jones, 1999), induced T cell-mediated immune responses that controlled virus replication after treatment interruption in SIV-infected macaques (Lifson *et al*, 2000; Lori *et al*, 2000) and in some HIV-infected individuals (Lisziewicz *et al*, 1999; Rosenberg *et al*, 2000); (ii) in contrast, chronic infection, where DC are not the major APC, is characterized by functionally impaired HIV-specific T cells (Lieberman *et al*, 2001) that could account for the lack of T cell-mediated viral clearance and rebound of virus replication during treatment interruption (Bonhoeffer *et al*, 2000; Miller *et al*, 2000; Deeks *et al*, 2001; Garcia *et al*, 2001; Lori and Lisziewicz, 2001; Ruiz *et al*, 2001); (iii) during established infection, cell-mediated immune responses suppress SIV/HIV (Bagnarelli *et al*, 1994; Cao *et al*, 1995; Klein *et al*, 1995; Harrer *et al*, 1996; Rosenberg *et al*, 1997; Ogg *et al*, 1998; Lori *et al*, 1999; Pitcher *et al*, 1999; Pontesilli *et al*, 1999; Schmitz *et al*, 1999), whereas humoral immune responses do not appear to play a major role in viral control (Wei *et al*, 2003).

The design of DermaVir as a therapeutic vaccine is distinct from most DNA vaccines developed for the prevention of SIV/HIV infection in the following ways: (i) the DNA construct mimics viral gene expression; (ii) epitopes derived from the DNA antigen are presented by DC in the lymph nodes; (iii) the vaccine is applied topically; and (iv) the vaccine induces Th-1 polarized HIV-specific T cells.

Results

Novel plasmid DNA in DermaVir for broad antigen presentation Since our goal was to develop a vaccine for the treatment of HIV, we wanted to use a DNA construct that mimics the expression of the wild-type virus in DC as it occurs during primary infection (Rowland-Jones, 1999). HIV-LTR-driven viral protein expression is efficient and does not require codon optimization. More importantly, it results in processing of most viral proteins and presentation of T cell epitopes, which is a key feature of the DermaVir vaccine. It has been recently demonstrated that DNA vaccination induced only a few epitopes that were identical to epitopes induced by the wild-type virus (Vogel *et al*, 2002). We have previously demonstrated that integrase-defective HIV-1 can express in DC but is not able to establish a productive infection, replicate, and integrate (Lisziewicz *et al*, 2001). To further characterize DermaVir in a relevant primate model we constructed a similar integrase mutant plasmid, pSHIV(int-), encoding a mutant pathogenic simian human immunodeficiency virus (SHIV 89.6P) (Reimann *et al*, 1996; Karlsson *et al*, 1997). This plasmid DNA can express both regulatory (e.g. tat, rev, nef) and structural proteins (e.g. gag, env), and is therefore expected to induce T cell-mediated immune responses with broad specificity (Fig 1).

Application of DermaVir DermaVir is applied topically to the surface of the skin after an exfoliation procedure. Shaving and exfoliation removes part of the stratum corneum, which rapidly regenerates. LC are located in the epidermis under the stratum corneum as a network of immune sentinels approximately 1000 cells per mm^2 to protect the body against pathogens that might enter upon injury to the skin. Disruption of the stratum corneum is required for the penetration of DermaVir to the epidermis and targeting of LC. The migration of LC is induced either by DermaVir application, which mimics a pathogen, or by the cytokines secreted by nearby keratinocytes upon exfoliation of the skin, or both. These "danger" signals are thought to trigger the LC to leave the epidermis and migrate via the lymphatic vessels to the draining lymphoid organs (Dieu-Nosjean *et al*, 1999).

Histological and macroscopic evaluation of DermaVir vaccination in non-human primates demonstrated that the skin exfoliation we developed for topical DermaVir administration is associated with the removal of the stratum corneum and preservation of the epidermis (Fig 2*a–d*). In such circumstances, it is unlikely that DermaVir would diffuse to the dermis and target dermal DC; however, we do not rule out this possibility. From the safety point of view, the primate study showed mild and transient erythema associated with skin preparation (Fig 2*e–g*), and lack of systemic toxicity. The safety results in primates were supported by a study

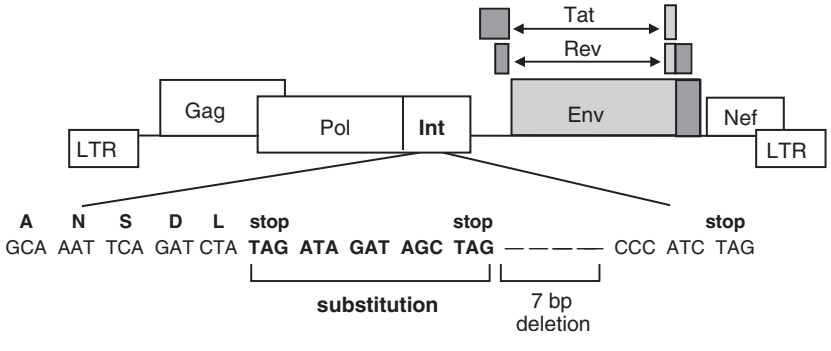

Figure 1
The plasmid DNA of DermaVir. Map of the plasmid DNA, pSHIV(int-), used for DermaVir vaccination in rhesus macaques. A mutation in the integrase gene has molecularly inactivated the full-length proviral DNA. Such a plasmid DNA is capable of effective gene expression upon transfection of 293T cells (data not shown). Virus replication is completely impaired in the absence of integration. LTR, gag, pol, and nef sequences originate from SIVmac239 (no fill). Env, tat, and rev genes originate from HIV-1. Env and second exons of tat and rev (*light gray*) are from the 89.6p isolate of HIV-1; others (*dark gray*) are from HXBc2.

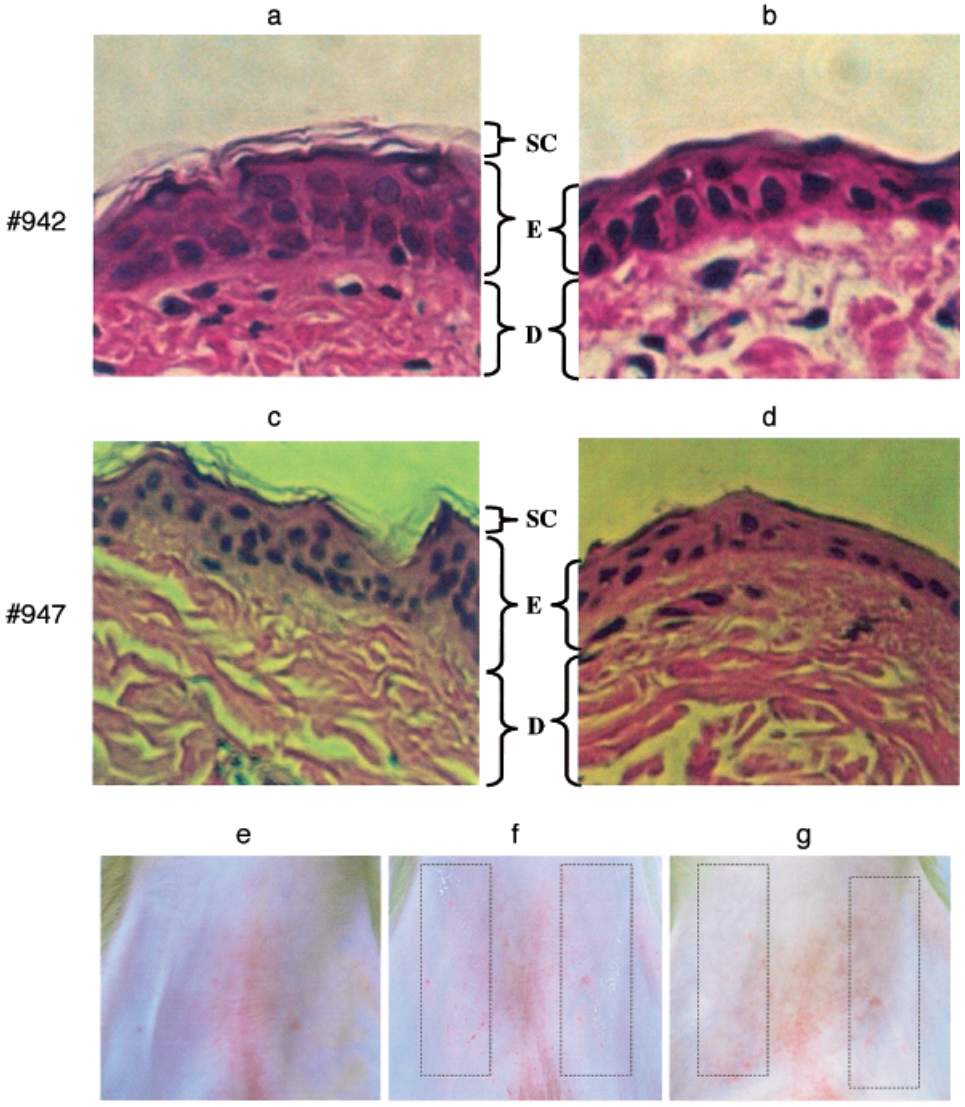

Figure 2
Topical application of DermaVir. DermaVir is applied to the surface of the skin after a skin preparation procedure causing the disruption of the stratum corneum by exfoliation that allows the vaccine to access the epidermis. Exfoliation is performed by rubbing the skin 50 times with an exfoliation sponge (3M, Heavy Duty). There is no hemorrhage, and in most cases mild and transient erythema is observed that is associated with skin preparation, not administration of DermaVir. (*a–d*) H&E-stained skin biopsies of two rhesus macaques, #942 and 947, prior to (*a*, *c*) and after (*b*, *d*) skin preparation. The typical local reaction after topical DermaVir administration demonstrated on clinical pictures of a rhesus macaque (*e–g*). The inguinal region before exfoliation (*e*), immediately following exfoliation and DermaVir application (*f*) (two treatment areas shown in the box), and 15 min post-DermaVir application (*g*). SC, stratum corneum; E, epidermis; D, dermis.

performed in swine, an accepted animal model for treatments administered via the skin, as porcine skin is similar in structure to human skin. Local reaction was limited to mild transient erythema that did not occur every time. There was no systemic toxicity, measured by blood chemistry and hematology, associated with DermaVir (data not shown).

Mechanism of DermaVir vaccination To activate naïve T cells, LC must undergo maturation when exposed to antigens and migrate to the draining lymph node. Therefore, we examined DermaVir-derived gene expression using RT-PCR in the draining lymph nodes after topical DermaVir vaccination in two different murine models; BALB/C and C57BL/6 (Table I). DermaVir was formulated with Gag-delta8.2, designated as pGag instead of the pSHIV(int-), because the LTR promoter does not result in gene expression in mice. pGag was a suitable reporter gene because gag mRNA can be quantified by a quantitative RT-PCR assay (Bagnarelli *et al*, 1994). Control animals were similarly treated with DermaVir formulated with pGFP. We found over 30,000 copies of gag mRNA per 10^6 cells in the inguinal lymph node of both mice species after vaccination with DermaVir. In contrast, we found less than 4,000 copies of gag mRNA per 10^6 cells in the lymph nodes of mice vaccinated with the same formulation containing a control DNA. This value was similar to that found in several untreated control mice (average of 4,536; SD = 1,610). We suspect that the gag-specific RT-PCR reaction gives a background in the different murine models, probably because of interference from endogenous retroviruses that are common in mice.

Table I. Detection of HIV-1 Gag mRNA by RT-PCR in the lymph nodes of mice after DermaVir vaccination

Animal	DNA in DermaVir	(gag)copies per 10^6 cells
BALB/C	pGag	32,800
BALB/C	pGFP	<5000
C57BL/6	pGag	64,104
C57BL/6	pGFP	<5000

Average control RT-PCR in different species of untreated mice was 4536 (SD = 1610). Values represented as <5000 copies are not different from the negative control mice.

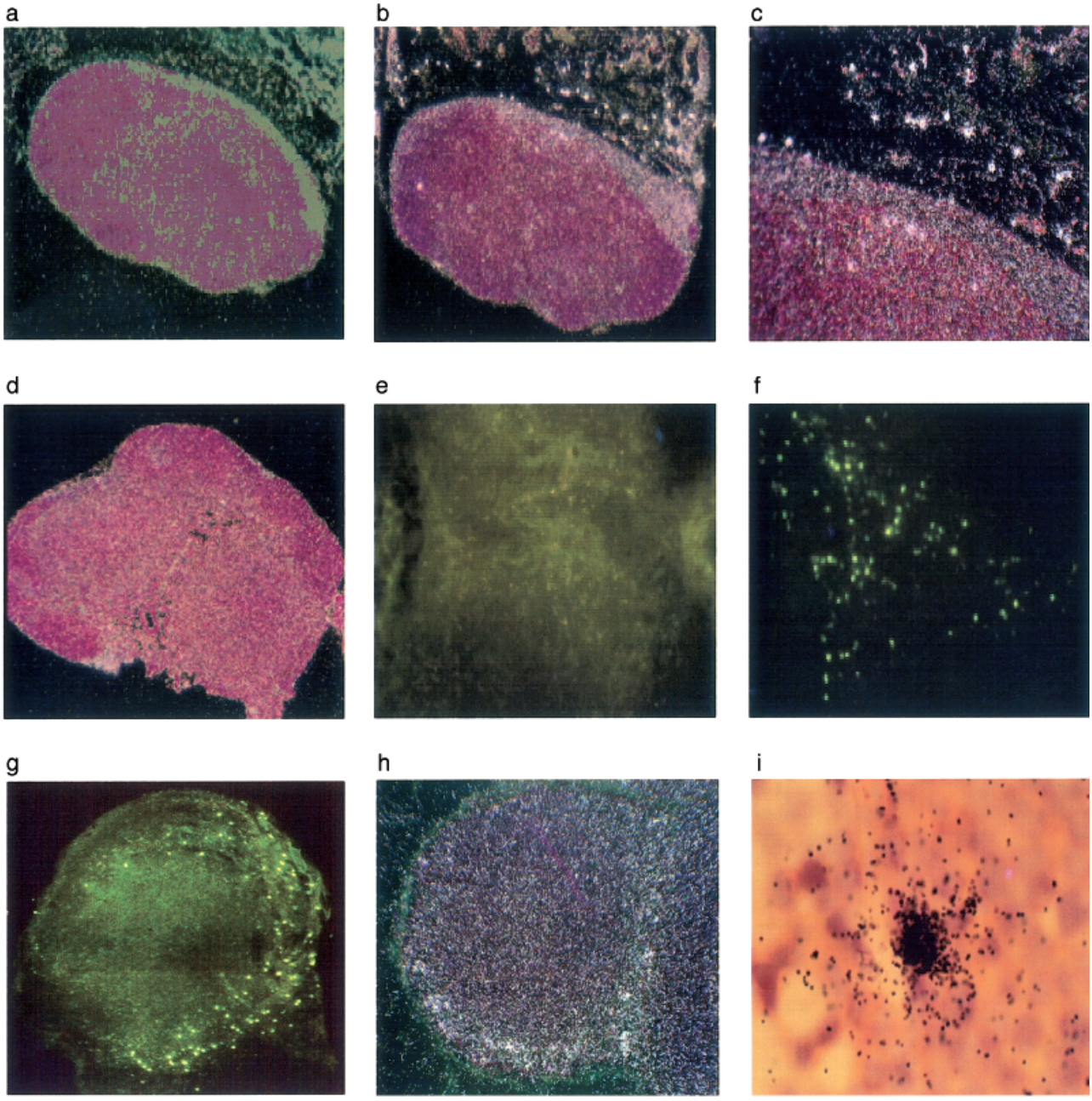

Figure 3
Gene expression in the lymph node after topical DermaVir vaccination in mice and monkeys. DermaVir was formulated with reporter plasmid DNA and applied topically on the skin of mice (representative of two experiments). (*a*) *In situ* hybridization using a 35S-labeled Neo-specific sense probe (negative control) 24 h after DermaVir application. No positive cells were detected by control *in situ* hybridizations. (*b*) *In situ* hybridization using a 35S-labeled Neo-specific antisense probe on a parallel section of (*a*). Transduced cells expressing plasmid DNA-derived genes (white silver grains over the cells). (*c*) Enlargement of (*b*). (*d*) *In situ* hybridization with a 35S-labeled Neo-specific antisense probe of a lymph node isolated from a naïve mouse (negative control). (*e–g*) Immunohistochemical staining with HIV Gag-specific antibody (KC57 FITC, Coulter, Florida) of a mouse lymph node 72 h post-immunization. DermaVir was formulated with plasmid DNA-expressing gag. (*e*) Isotype control of p55 antibody (15 s exposure). (*f*) p55 antibody staining (5 s exposure) on a parallel section of (*d*). (*g*) Protein expression is localized in the paracortical region of the lymph node. Quantification of these cells in mice revealed an average of 222 (30–400) Gag-expressing cells per 0.05 mm sections, corresponding to an average of 68 positive cells per mm^2. Parallel sections stained with the isotype control gave an average of 0.6 (0–2) positive cells per section. Representative experiments, repeated at least three times. (*h*) Gene expression in lymph node dendritic cells (DC) after topical DermaVir vaccination of rhesus macaques (same methods as in mice (*a–c*)). Dark-field microscopic image of cells showing (*white*) silver grains over positive cells. Control hybridization of a parallel section showed no positive cells (not shown). (*i*) A single DC expressing Neo gene encoded by the DNA used for DermaVir formulation. Black dots are silver grains (*in situ* hybridization) demonstrating plasmid DNA-derived gene expression. The section was also stained with p55 antibody (anti-human Fascin, 55K-2, Dako Corp. Carpenteria, California) which is a marker for lymph node DC (*brown* in the figure). Quantitative analysis in macaques revealed 153 DC expressing DNA per 13.4 mm^2 total analyzed sections (average 11 positive cells per mm^2).

To visualize the RNA-expressing cells in the lymph nodes of DermaVir-vaccinated mice, *in situ* hybridization experiments were carried out, using another reporter DNA construct encoding the neomycin-phosphotransferase gene (Neo). Figure 3*a–d* demonstrate the DNA-expressing cells migrating into the lymph node 24 h after topical DermaVir

vaccination. As demonstrated in Fig 3*a* and *d*, *in situ* hybridizations of parallel sections using a control sense probe and sections of control mice hybridized with the antisense probe did not reveal any positive cells. Quantitative analysis revealed 153 DC expressing DNA per 13.4 mm^2 total analyzed sections (average 11 positive cells per mm^2). These *in situ* hybridization results supported the RT-PCR data (Table I) and suggested that topical DermaVir vaccination can directly transduce large numbers of skin-derived cells in the lymph node.

To visualize protein expression, we formulated DermaVir with pGag and analyzed the lymph nodes with an HIV Gag-specific antibody (Fig 3*e–g*). Positive cells were mainly located in the paracortical area, which is also referred to as the T cell area known to contain the migrating LC-derived DC. Altogether, these results further confirmed the RT-PCR data (Table I) and demonstrated that topical DermaVir vaccination results in gene expression in the lymph node.

Since the murine epidermis differs from that of primates (Foster *et al*, 1990; Matsue *et al*, 1993), we performed the same experiment in rhesus macaques. Our analysis confirmed that topical DermaVir vaccination results in DNA-expressing cells located in the T cell area of the lymph node (Steinman *et al*, 1997), specifically in the paracortical region (Fig 3*h*). The identity of these positive DNA-expressing DC was demonstrated with antibody staining specific for DC in the lymph node (anti-human Fascin, 55K-2, Dako Corp., Carpenteria, California) (Steinman *et al*, 1997) (Fig 3*i*). Control hybridization of a parallel section with the sense probe did not detect positive cells (not shown). Quantitative analysis revealed 153 DC expressing DNA per 13.4 mm^2 total analyzed sections (average 11 positive cells per mm^2). The number of gene-expressing DC after topical DermaVir vaccination was quite comparable with the number of gene-expressing DC detected previously in the lymph node after *ex vivo* administration of DermaVir-transduced DC (Lisziewicz *et al*, 2001). These results indicated that after topical DermaVir vaccination gene expression in lymph node DC could be achieved in non-human primates.

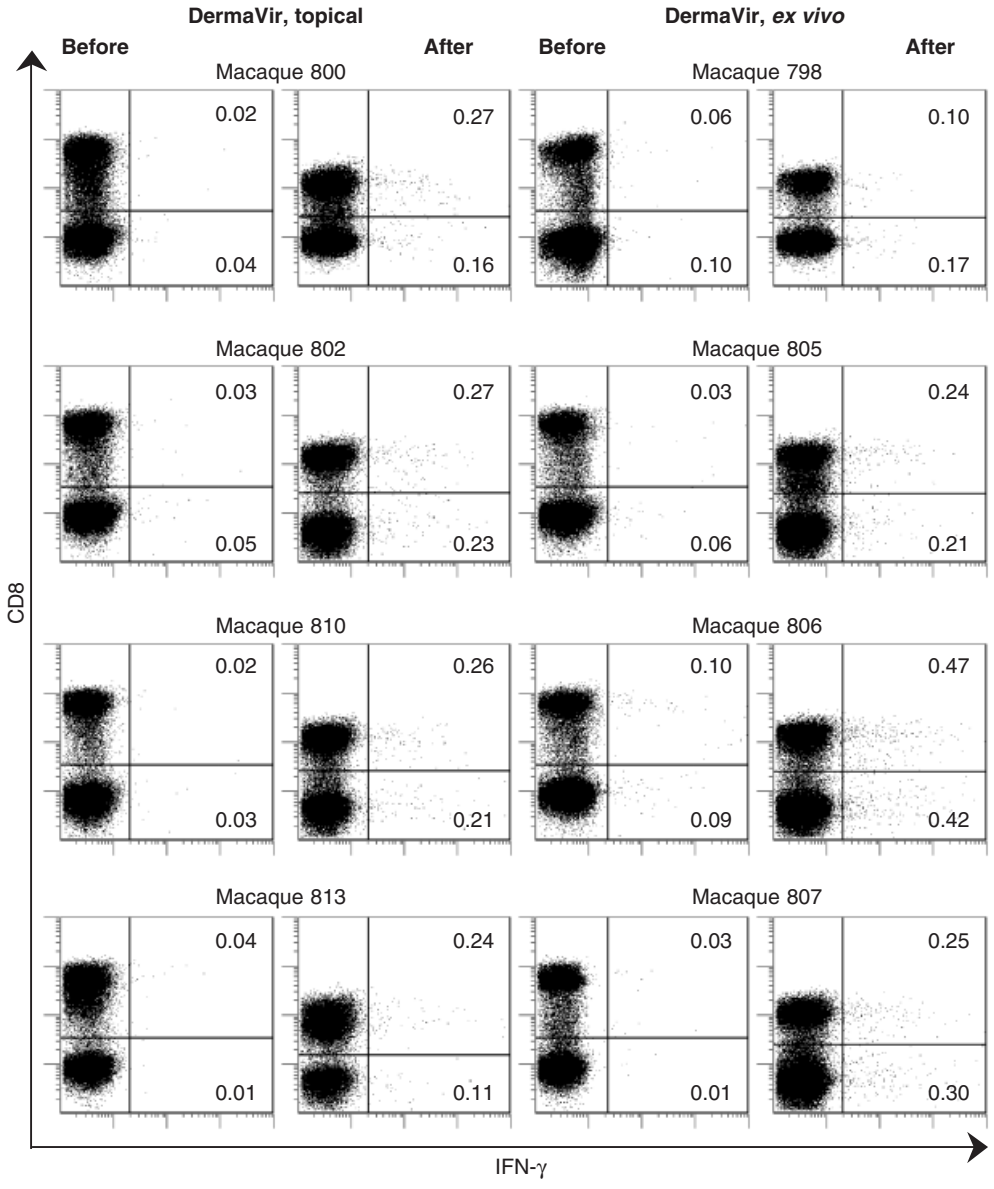

Figure 4
Induction of simian immunodeficiency virus (SIV)-specific T cells by DermaVir immunization. SIV-specific T cell responses were detected in the peripheral blood of macaques prior to and 3 wk after the first topical (*left two columns*) and *ex vivo* (*right two columns*) DermaVir immunization. Histograms illustrate SIV-specific T cells measured as IFN-γ expression in $CD8^+$ and $CD8^-$ (gated on CD3) T lymphocytes after stimulation with whole chemically inactivated SIV antigen (Lori *et al*, 2000; Xu *et al*, 2002). Numbers represent the percentages of IFN-γ expressing $CD8^+$, $CD3^+$, and $CD8^-$, $CD3^+$ T cells in the quadrates.

Immune responses induced by DermaVir We evaluated DermaVir-induced immune responses in naïve rhesus macaques, since this is a suitable animal model to study immune-based interventions for HIV. For these studies, DermaVir was formulated with our prototype therapeutic plasmid DNA, pSHIV(int-) (Fig 1), that is based on a SHIV construct. Four monkeys were immunized topically and another four via *ex vivo* immunization with DermaVir-transduced DC as described previously (Lisziewicz *et al*, 2001). SIV-specific T cells were measured in the peripheral blood after whole SIV viral antigen stimulation by interferon (IFN)-γ production (Fig 4). The average frequency of SIV-specific $CD8^+$, $CD3^+$, and $CD8^-$, $CD3^+$ T cells prior to immunization was 0.06% and 0.07% (SD = 0.03 and 0.04), respectively, a characteristic background in naïve macaques. After one immunization all the animals developed SIV-specific T cell responses. The average frequency of SIV-specific $CD8^+$, $CD3^+$, and $CD8^-$, $CD3^+$ T cells in the topically immunized group was 0.26% and 0.18% (SD = 0.01 and 0.05), respectively. The average frequency of SIV-specific $CD8^+$, $CD3^+$, and $CD8^-$, $CD3^+$ T cells in the *ex vivo* immunized group was 0.27% and 0.28% (SD = 0.15 and 0.11), respectively. These results demonstrate that topical DermaVir immunization was able to induce antigen-specific CD8 and CD4 T cell-mediated immune responses in non-human primates similar to *ex vivo* DC vaccination.

Vaccinations were administered at months 0, 2, 5, 13, and 21. Despite repeated immunizations, antibody responses measured after every vaccination were undetectable by ELISA in all of the animals, even after the fifth DermaVir vaccination. After the first vaccination, we consistently detected a transient increase of SIV-specific T cells, peaking 3 wk after vaccination in the peripheral blood; however, 8 wk after vaccination the frequency of SIV-specific cells was undetectable in the intracellular cytokine assay. Interestingly, after the third vaccination we only sporadically detected a very low amount of SIV-specific T cells in the peripheral blood of the animals. Meaningful T cell responses with the ELISPOT assay were not detected.

To confirm the induction of Th-1-type cell-mediated immune responses by DermaVir, an *in vivo* assay was used, similar to that used to determine exposure to *Mycobacterium tuberculosis*. When people have been exposed to tuberculosis, a cell-mediated immunity develops that can be detected as a local response after intradermal injection of a small amount of tuberculin. These delayed-type hypersensitivity (DTH) responses are mediated by T cells, because they can be seen in individuals who lack immunoglobulins. To perform the skin test, we used the same chemically inactivated SIV (Arthur *et al*, 1998) that we used for stimulation of T cells to detect SIV-specific T cells in the peripheral blood. As control antigen, we used the supernatant of the parental cell line that was utilized to produce the SIV (called microvesicles). The skin test was performed 5 mo after the fourth vaccination, when SIV-specific T cell responses were undetectable by intracellular cytokine assay in the peripheral blood. All monkeys immunized either topically or *ex vivo* with DermaVir developed DTH responses to SIV antigen but not to the microvesicle control antigen (Fig 5). The reactive DTH tests in the immunized macaques further substantiated the activation of T cell-mediated immune responses by DermaVir and suggested the presence of a memory T cell population in tissue reservoirs that can expand upon antigenic stimulation.

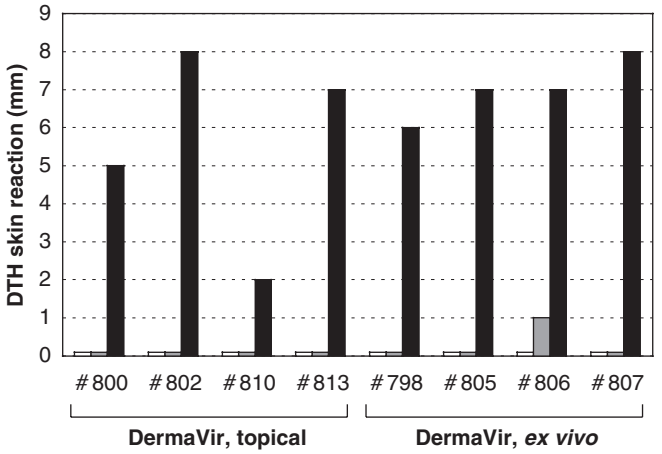

Figure 5
Delayed-type (type IV) simian immunodeficiency virus (SIV)-specific hypersensitivity (DTH) responses in macaques immunized topically and *ex vivo* with DermaVir. DTH skin reactions (diameter, millimeters) were detected in the skin of macaques 25 wk after the fourth topical (animals 800, 802, 810, and 813) and *ex vivo* (animals 798, 805, 806, and 807) DermaVir immunization. DTH reactivity was measured 72 h post-intradermal injection with 100 μL normal saline (*empty bars*); 100 μL control microvesicles (*gray bars*), representing the supernatant of the parental cell line that was utilized to produce the SIV antigen; and 100 μL (2 μg of p27) chemically inactivated SIVmac239 antigen (Arthur *et al*, 1998) (*black bars*).

Discussion

DermaVir is a novel DNA immunization method designed to improve antigen presentation and induce cytotoxic T cell responses for the treatment of HIV/AIDS. DermaVir is applied directly to the epidermis above the basal keratinocytes to penetrate the skin surface and reach a network of sentinels that serve to initiate immune responses against pathogens. After DermaVir immunization, plasmid-derived gene expression occurred in lymph node DC. Gene-expressing DC induced T cell-mediated immune responses. Our experiments did not demonstrate cross-presentation of antigens that might also occur. In naïve macaques, DermaVir effectively primed antigen-specific CD4 helper and CD8 T cells producing intracellular IFN-γ after *in vitro* antigenic stimulation. Boosting with DermaVir did not increase T cell responses in macaques, similar to recent observations in DC-immunized mice (Ludewig *et al*, 2001). The possible explanation for these findings is that DC boosting reactivates memory cytotoxic T lymphocytes (CTL) that can rapidly eliminate the antigen-presenting DC and thereby limit the booster effect, an important mechanism to avoid exaggerated CTL responses. But T cell memory was maintained in the reservoirs, even when SIV-specific T cell frequency was under the limit of the detection in the peripheral blood (0.1%), as shown by the DTH responses to SIV antigens in all the animals immunized either topically or *ex vivo* with DermaVir. The reactive DTH test and lack of humoral response observed after repeated DermaVir immunizations

are similar to that observed in individuals previously exposed to *M. tuberculosis* who do not develop antibody responses and have reactive tuberculin DTH tests. Interestingly, recent studies demonstrated that in *M. tuberculosis* infection DC, which are not permissive for the growth of the bacteria, take up the bacteria and induce cellular immune responses (Geijtenbeek *et al*, 2003; Tailleux *et al*, 2003). These results are in line with the concept that expression of antigens within DC will generate polarized cellular responses. The fact that DNA-expressing DC in the lymph node did not induce antibody production, regardless of the topical or *ex vivo* DermaVir vaccination methods, could be explained by selective antigen expression in DC in the absence of significant antigen expression in the local somatic cells (e.g. myocytes, keratinocytes, and fibroblasts), which serve as antigen reservoirs for conventional DNA vaccines (Tuting *et al*, 1998; Akbari *et al*, 1999).

We have shown here in a primate model that topical DermaVir vaccination is comparable with *ex vivo* DC-based vaccination. DermaVir, administered topically or *ex vivo*, resulted in a similar number of transduced DC in the lymph node and induced a similar quantity and quality of SIV-specific Th1-type T cell responses. Both modalities of DermaVir administration specifically utilize DC, cells capable of converting naïve T cells to functional cytotoxic T cells and polarizing the immune system towards T cell-mediated immune responses. Since the immune system of HIV-infected individuals has already been primed during primary infection by large amounts of viral antigen, the purpose of DermaVir therapy is to improve the presentation of viral antigens in order to induce functional T cell responses that can control viral replication. It is indeed unlikely that HIV-specific antibodies will have a role in the control of viral load during established infection (Richman *et al*, 2003; Wei *et al*, 2003).

Our experiments demonstrated a remarkably high efficiency of topical gene transfer to lymph node DC. In the mouse model the efficacy was between 30 and 90 cells per mm^2 and in the primate model 11 cells per mm^2 (Fig 3). The disparity of these numbers might reflect the differences between the murine and primate epidermis and lymph nodes. Since the average diameter of a lymph node is, however, about 1.8 and 6 mm in mice and monkeys, respectively, when the size of the lymph node was taken into account, a conservative estimate of the total number of positive cells per lymph node in the murine model was between 1,000 and 4,000 cells, whereas in the primate model it was about 20,000 cells.

Conventional DNA vaccines designed for prevention of HIV/SIV infection usually require the optimization of protein expression to provide a large amount of viral antigens and thereby induce both antibody and T cell responses in hosts who have not encountered such antigens. Generally, these DNA constructs contain one or more codon-optimized HIV genes driven by a strong constitutive promoter (Barouch and Letvin, 2000; Corbet *et al*, 2000; Robinson *et al*, 2002). Second-generation plasmid DNAs have been optimized for high-level expression in cell lines and improved expression has been shown to be associated with more efficient induction of immune responses. The plasmid DNA in DermaVir was designed to express all viral genes in DC except integrase in order to induce HIV-specific T cells with broad specificity. HIV-LTR-driven viral protein expression does not require artificial, codon-optimized sequences or heterologous promoters introduced in other DNA vaccines to achieve efficient expression (Robinson *et al*, 2002).

In DermaVir the DNA was formulated with a cationic polymer (PEIm) in glucose. The cationic polymer complexes the DNA, forming a small mannosilated particle and the glucose stabilizes the complex by inhibiting aggregation prior to the vaccine application. The DermaVir particle contains pathogen-associated molecular patterns (e.g. mannose from PEIm and CpG motifs from the plasmid DNA), and therefore might be recognized by toll like or other receptors on LC (Akira *et al*, 2001). PEIm plays an important role after receptor-mediated endocytosis of DermaVir by breaking the endosome (Boussif *et al*, 1995) and facilitating the trafficking of the DNA into the nucleus, where the DNA-encoded antigens are expressed (Pollard *et al*, 1998).

Antigen-presenting DC in the lymph node induce T cell-mediated anti-tumor immune responses (Banchereau and Steinman, 1998; Palucka and Banchereau, 1999; Schadendorf and Nestle, 2001). Here, we extended these observations to DC expressing viral DNA-derived antigens following topical DermaVir vaccination. These results confirm and expand the concept of using DC as preferential targets to elicit cellular immune responses (Kirk and Mule, 2000; Schadendorf and Nestle, 2001). The technology described here could provide the basis for novel DNA-based medicines. Various plasmids could be constructed to obtain the expression of a wide variety of tumor and viral antigens by DC in the lymph nodes in order to induce antigen-specific T cell responses. Immune responses might be augmented by the co-expression of recombinant cytokines (Ahuja *et al*, 1999; Melero *et al*, 1999; Ozawa *et al*, 1999; Takayama *et al*, 1999). The technology could allow genetic manipulation of DC to express IL-10, TGF-β, FasL, and CTLA4Ig, for example, which have been suggested to enhance tolerance and allograft survival (Lu *et al*, 1999). In addition, the techniques described here might be used as tools to elucidate as yet unanswered questions in immunology, such as the role of lymph node DC in antigen presentation and immune induction.

Others have demonstrated inhibition of SIV replication (Lu *et al*, 2003) with DC-based *ex vivo* therapeutic immunization; however, these *ex vivo* techniques are cumbersome, expensive, and limited to highly specialized laboratories. DermaVir represents a significant advancement in the field of DC-based therapeutic immunization: (i) it can be manufactured for large-scale human use; (ii) it utilizes a needle-free, topical application; and (iii) it only requires a small amount of DNA. (iv) All pre-clinical studies with DermaVir show no serious safety concerns. Furthermore, (v) DermaVir suppression of viral load during chronic SIV infection in non-human primates (Lisziewicz *et al*, in press) has recently been observed. These features make DermaVir a promising new approach to the treatment of HIV/AIDS.

Materials and Methods

Plasmid DNA used for DermaVir formulation A plasmid, known to express green fluorescent protein and neomycin phosphot-

ransferase, pGFP (Clonetech, Palo Alto, California), was utilized in most of the experiments demonstrating the mechanism of DermaVir vaccination. A CMV-driven plasmid expressing the HIV-1 gag protein, Gag-delta8.2 (Naldini *et al*, 1996), was kindly provided by Inder Verma. For primate immunology experiments the prototype DermaVir plasmid DNA, expressing a replication-defective virus, was used. The full-length, but integration defective, SHIV plasmid pSHIV(int-), created by stepwise strategy starting with p-5′SHIV (clone KB9) and p-3′SHIV (clone 64/KB9) provided by Joseph Sodroski of Harvard University, was used in $DermaVir_{SHIV}$ for all primate experiments. These clones, derivatives of SHIV 89.6, are also available from the NIH Research and Reference Reagent Program. First, a clone of p-5′SHIV with the deletion of the internal *Bgl*2–*Bgl*2 sites located in the pol gene was created and termed p-5′SHIV(dBg). The internal *Bgl*2–*Bgl*2 fragment was mutated by PCR amplification of the fragment with primers introducing mutations with stop codons and cloned into a separate vector and termed pBg08. The mutated fragment was isolated and inserted into p-5′SHIV(dBg) to obtain p-5′SHIV(int-). The *Xho*1–*Sph*1 viral fragment (~6.5 kb) from p-5′SHIV(int-) and *Sph*1–*Not*1 viral fragment (~4.0 Kb) from p-3′SHIV clones were isolated and cloned into a pBluescript (Stratagene Inc., La Jolla, California) vector backbone to obtain the pSHIV(int-) clone. The sequence of the junctions and of the integrase gene region of this clone was checked. It contained small deletions, frame shift, and three separated stop codons in the integrase gene open reading frame. It also contained stop codons in the other reading frame in this region. SIV_{mac239} sequence: (nt 4696) 5′-AGATCTAGGGACTTG GCAAATGGATTGTACCCAT-3′ (nt 4729). pSHIV(int-) sequence: 5′-AGATCTATAGATAGATAGCTAGCCCAT-3′.

Topical and *ex vivo* DermaVir immunization DermaVir is formulated to make a approximately 100 nm particle containing DNA, PEIm, and glucose and the particle size was determined by light scattering using a Zetasizer (Malvern Instrument, Orsay, France). For topical DermaVir application, a skin preparation procedure was developed to allow vaccine penetration to the epidermis. Hair was removed by shaving and the skin was exfoliated with an exfoliating sponge (3M Scotch-Brite Heavy Duty Scrub Sponge 3M Corp., St. Paul, Minnesota), followed by tape stripping (Fig 2). This procedure results generally in transient and mild erythema but not in hemorrhage and eschar formation. 0.2 mL DermaVir was applied on approximately 40 cm^2 prepared skin at four locations: the left and right upper inguinal region and left and right axillary region. After 30 min of contact time under general anesthesia, DermaVir solution dried and the animals were returned to the cage. For mice, 0.2 mL DermaVir was applied on approximately 20 cm^2 area on the dorsum. In all *ex vivo* experiments, 10^6 *ex vivo* DermaVir-transduced DC were injected subcutaneously at the same four locations as previously described (Lisziewicz *et al*, 2001).

Animals All animal experiments were performed under protocols approved by an Institutional Animal Care and Use Committee. Four- to 6-wk-old female BALB/C mice were used. The mice were anesthetized using methoxyflurane. In non-human primate studies, rhesus macaques were sedated with ketamine xylazine and placed on a circulating water heating pad for the duration of the immunization procedure. For the *in situ* hybridization experiment, the draining lymph node was surgically removed 24 h after DermaVir vaccination.

***In situ* hybridization** *In situ* hybridization was conducted using standard protocols (Fox and Cottler-Fox, 1993a, b). Riboprobes were ^{33}P labeled and were determined to detect 20–30 copies per cell of HIV gag RNA, although in the case of Neo probes the sensitivity was somewhat less. The slides were exposed for 5 d before development and examination by dark-field microscopy. Immunohistochemistry was performed using protocols recommended by the supplier of the primary antibodies.

RT-PCR RNA was isolated by using a TRIzol reagent (GIBCO BRL, Gaithersburg, Mary Land) according to the protocol of the manufacturer. Quantitative RT-PCR were performed with the Roche kit, detection limit of 200 copies of HIV-1 RNA (Roche Diagnostic Systems Inc., Branchburg, New Jersey.)

Quantitative determination of SIV-specific T cell responses in peripheral blood The assay was performed as previously described (Lori *et al*, 2000; Xu *et al*, 2002). PBMC were plated in round-bottom 96-microtiter plates (Corning Inc., Corning, New York) at 0.5 million cells per well in 0.1 mL complete RPMI-1640 medium containing 5 μg Zn-finger-inactivated SIV (kindly provided by Jeff Lifson, NCI, Frederick, Mary Land) or mock antigen and 50 IU per mL rhIL-2 (gift from Hoffman La Roche). Cells were cultured for 15 h and treated with Brefeldin A (Sigma, St Louis, Missouri) at 10 μg per mL for an additional 3 h. Cells were collected and aliquoted into 0.5 million cells per test tube. After washing once with 2 mL phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), cells were suspended in 0.1 mL PBS/1% BSA and stained with CD8-Phycoerythrin-Cyanine 5 (PC5; Immunotech, Marseille, France) and CD3 fluorescein isothiocyanate (FITC; BD PharMingen, San Diego, California) fluorescent antibodies for 15 min at room temperature. After washing, cells were fixed with 2% paraformaldehyde, pH 7.4 for 10 min and washed with PBS/1% BSA, and then permeabilized with 0.1 mL 0.1% saponin in PBS/1% BSA for 5 min and stained with IFN-γ-R-Phycoerythrin conjugated (IFN-γ-PE; BD PharMingen) antibody for 15 min at room temperature. After intracellular staining, cells were washed twice with 1 mL PBS and then re-suspended in a 0.5 mL 1% paraformaldehyde PBS buffer. Samples were analyzed by FACS (EPICS XL-MCL, Coulter, Miami, Florida). A total of 50,000 events were acquired. Gated $CD3^+$ $CD8^+$ cells were examined for staining with IFN-γ.

Detection of SIV-specific T cell responses *in vivo* A skin test was developed to detect SIV-specific T cell responses *in vivo*. SIV and control antigens in 0.1 mL physiological salt were intradermally injected. DTH skin reactions were recorded as the diameter in millimeters by a blinded operator 72 h later. The following purified soluble antigens were used: 2 μg (p27) Zn-finger-inactivated SIV_{mac239} (Arthur *et al*, 1998); microvesicles as control (the supernatant of the SupT1 cell line used to produce SIV_{mac239}); and normal saline solution for suspension of antigens (Zinc-inactivated-SIV_{mac239} and microvesicles were kindly provided by Jeff Lifson, NCI).

Detection of antibody responses The native SIV p27 used was purified from conditioned media from infected mammalian cells (Hut 78) that secrete SIV env and gag proteins. The recombinant HIV-1 89.6p gp140 protein was prepared from (293) mammalian cells expressing the gene. To prepare ELISA plates, SIV p27 was added at 50 ng per well, and gp140 at 50 ng per well. Binding antibody ELISA titers are routinely presented as the reciprocal of the serum dilution at which the adsorbance of the test serum is twice that of negative control serum (Zhao *et al*, 2003; Patterson *et al*, 2004).

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